National Technical University of Athens

School of Mechanical Engineering

Systems Biology & Bioengineering Research Laboratory

Quantification of the concentration of Albumin protein in urine samples via bead-based multiplex ELISA

Tsiripidis Dimitrios
Tzanetos Konstantinos
Champsas Georgios

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1.Introduction

The enzyme-linked immunosorbent assay (ELISA) is a test that uses antibodies and color change to identify a substance.

ELISA is a popular format of "wet-lab" type analytic biochemistry assay that uses a solid-phase enzyme immunoassay (EIA) to detect the presence of a substance, usually an antigen, in a liquid sample or wet sample.

The ELISA has been used as a diagnostic tool in medicine and plant pathology, as well as a quality-control check in various industries.

Antigens from the sample are attached to a surface. Then, a further specific antibody is applied over the surface so it can bind to the antigen. This antibody is linked to an enzyme, and, in the final step, a substance containing the enzyme's substrate is added. The subsequent reaction produces a detectable signal, most commonly a color change in the substrate.

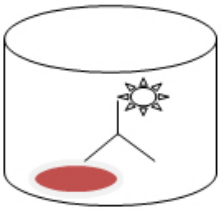
Performing an ELISA involves at least one antibody with specificity for a particular antigen. The sample with an unknown amount of antigen is immobilized on a solid support (usually a polystyrene microtiter plate) either non-specifically (via adsorption to the surface) or specifically (via capture by another antibody specific to the same antigen, in a "sandwich" ELISA). After the antigen is immobilized, the detection antibody is added, forming a complex with the antigen. The detection antibody can be covalently linked to an enzyme, or can itself be detected by a secondary antibody that is linked to an enzyme through bioconjugation. Between each step, the plate is typically washed with a mild detergent solution to remove any proteins or antibodies that are non-specifically bound. After the final wash step, the plate is developed by adding an enzymatic substrate to produce a visible signal, which indicates the quantity of antigen in the sample.

(https://en.wikipedia.org/wiki/ELISA ,2015)

2.Types of ELISA

2.1 Direct ELISA

An antigen coated to a multiwell plate is detected by an antibody that has been directly conjugated to an enzyme. This can also be reversed, with an antibody coated to the plate and a labeled antigen used for detection, but the second option is less common.

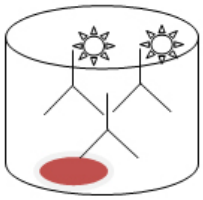


- Advantages:
  - Quick because only one antibody and fewer steps are used.
  - Cross-reactivity of secondary antibody is eliminated.

- Disadvantages:
  - Immunoreactivity of the primary antibody might be adversely affected by labeling with enzymes or tags.
  - Labeling primary antibodies for each specific ELISA system is time-consuming and expensive.
  - No flexibility in choice of primary antibody label from one experiment to another.
  - Minimal signal amplification.

2.2 Indirect ELISA

Antigen coated to a polystyrene multiwell plate is detected in two stages or layers. First an unlabeled primary antibody, which is specific for the antigen, is applied. Next, an enzyme-labeled secondary antibody is bound to the first antibody. The secondary antibody is usually an anti-species antibody and is often polyclonal.



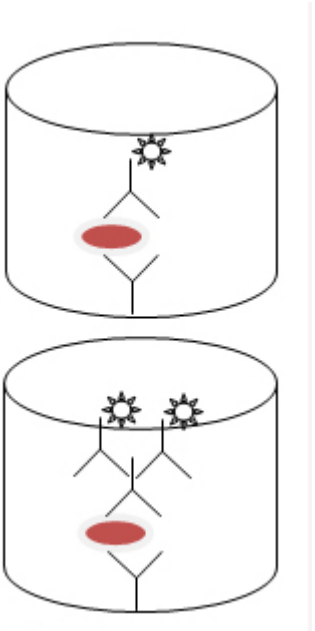
- Advantages:
  - A wide variety of labeled secondary antibodies are available commercially.
  - Versatile because many primary antibodies can be made in one species and the same labeled secondary antibody can be used for detection.
  - Maximum immunoreactivity of the primary antibody is retained because it is not labeled.
  - Sensitivity is increased because each primary antibody contains several epitopes that can be bound by the labeled secondary antibody, allowing for signal amplification.
  - Different visualization markers can be used with the same primary antibody.

- Disadvantages:
  - Cross-reactivity might occur with the secondary antibody, resulting in nonspecific signal.
  - An extra incubation step is required in the procedure.

2.3 Sandwich ELISA

Sandwich ELISAs typically require the use of matched antibody pairs, where each antibody is specific for a different, non-overlapping part (epitope) of the antigen molecule. The first antibody, termed the capture antibody, is coated to the polystyrene plate. Next, the analyte or sample solution is added to the well. A second antibody layer, the detection antibody, follows this step in order to measure the concentration of the analyte. Polyclonals can also be used for capture and/or detection in a sandwich ELISA provided that variability is present in the polyclonal to alow for both capture and detection of the analyte through different epitopes. If the detection antibody is conjugated to an enzyme, then the assay is called a direct

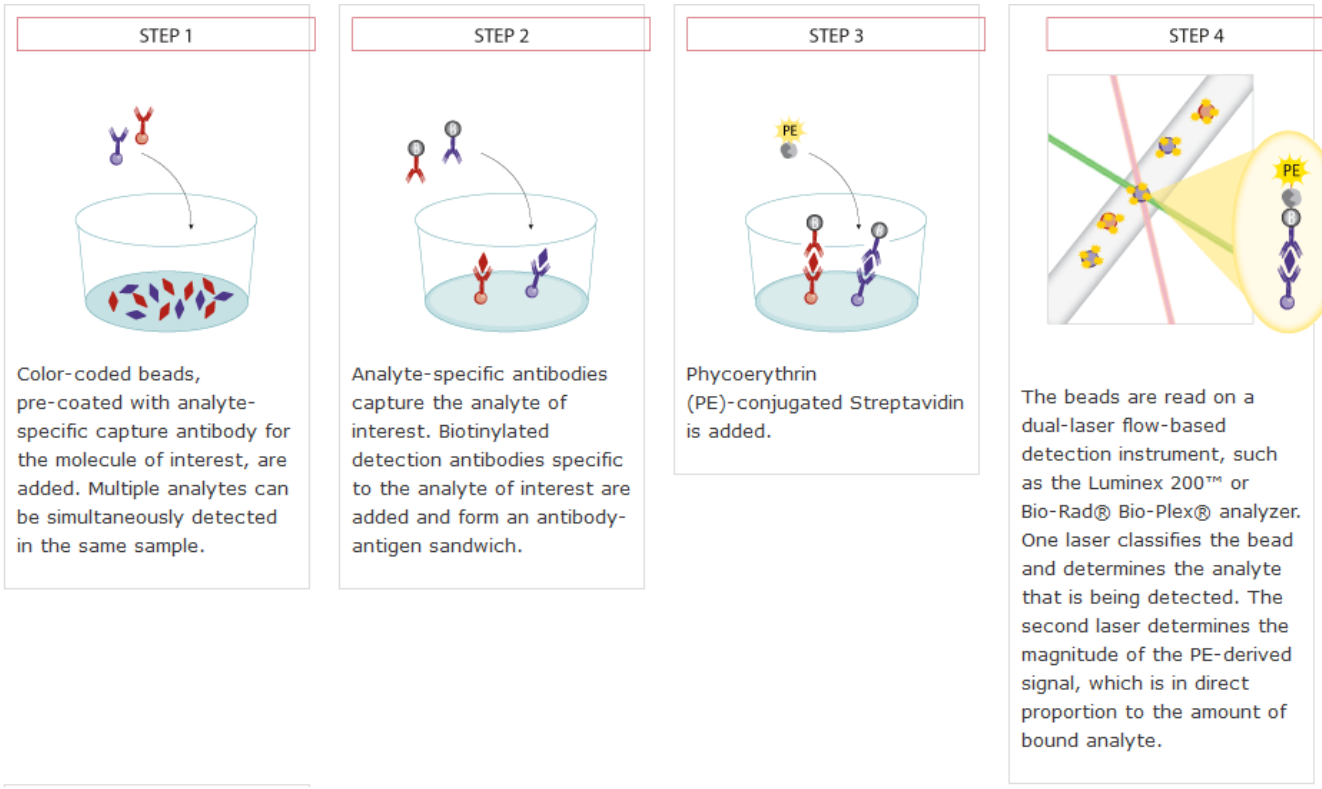
sandwich ELISA. If the detection antibody is unlabeled, then a second detection antibody will be needed resulting in an indirect sandwich ELISA

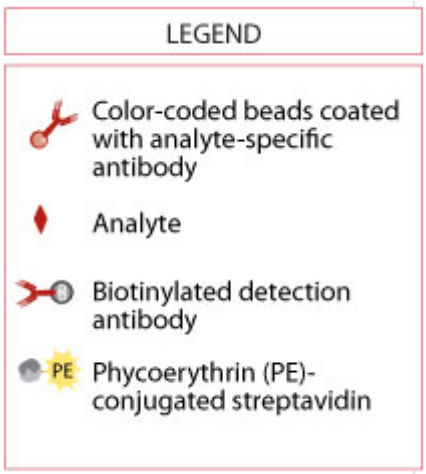


- Advantages:
  - High specificity, since two antibodies are used the antigen/analyte is specifically captured and detected
  - Suitable for complex samples, since the antigen does not require purification prior to measurement
  - Flexibility and sensitivity, since both direct and indirect detection methods can be used

(https://www.abdserotec.com/elisa-types-direct-indirect-sandwich-competition-elisa-formats.html ,2015)

- Bead Based Multiplex ELISA

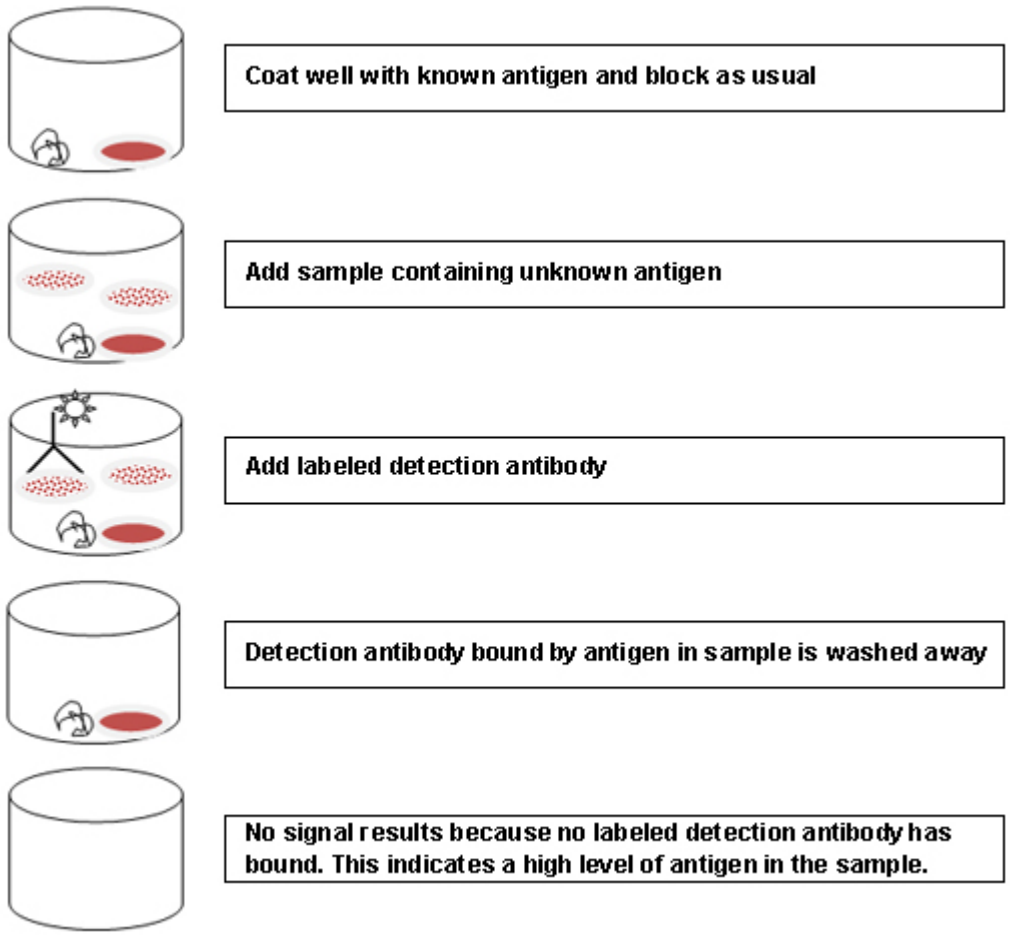




(https://www.rndsystems.com/resources/technical/luminex-bead-based-assay-principle ,2016)

2.4 Competitive ELISA

This is the most complex ELISA, and is used to measure the concentration of an antigen (or antibody) in a sample by observing interference in an expected signal output. Hence, it is also referred to as an inhibition ELISA. It can be based upon any of the above ELISA formats, direct, indirect, or sandwich, and as a result it offers maximum flexibility in set up. It is most often used when only one antibody is available to the antigen of interest or when the analyte is small, i.e. a hapten, and cannot be bound by two different antibodies. In this case samples are added to an ELISA plate containing a known bound antigen. After coating, blocking, and washing steps, unknown samples are added the plate. Detection then follows pretty much as with other ELISA formats. If the antigen in the sample is identical to the plate-adsorbed antigen, then there will be competition for the detection antibody between the bound and free antigen. If there is a high concentration of antigen in the sample, then there will be a significant reduction in signal output of the assay. Conversely, if there is little antigen in the sample, there will be minimal reduction in signal. Therefore, with a competition ELISA, one is actually measuring antigen concentration by noting the extent of the signal reduction. If the detection antibody is labeled, then this would be a direct competition ELISA and if unlabeled, then this would be an indirect competition ELISA.



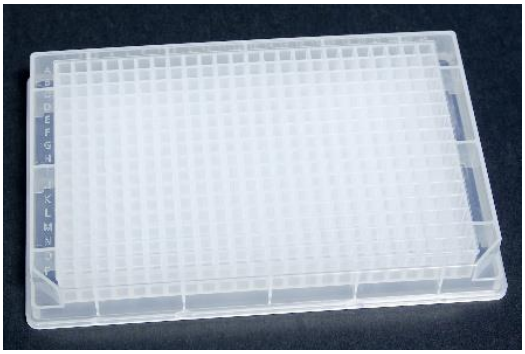
(https://www.abdserotec.com/elisa-types-direct-indirect-sandwich-competition-elisa-formats.html ,2015)

3.Lab Equipment

- Multiwell Plate:

  A flat plate with multiple "wells" used as small test tubes. The microplate has become a standard tool in analytical research and clinical diagnostic testing laboratories. A very common usage is in the enzyme-linked immunosorbent assay (ELISA), the basis of most modern medical diagnostic testing in humans and animals.A microplate typically has 6, 24, 96, 384 or even 1536 sample wells arranged in a 2:3 rectangular matrix. Some microplates have even been manufactured with 3456 or even 9600 wells, and an "array tape" product has been developed that provides a continuous strip of microplates embossed on a flexible plastic tape.

  (https://en.wikipedia.org/wiki/Microtiter_plate)



- Pipette:

  A pipette or dropper is a laboratory tool commonly used in chemistry, biology and medicine to transport a measured volume of liquid, often as a media dispenser. Pipettes come in several designs for various purposes with differing levels of accuracy and precision, from single piece glass pipettes to more complex adjustable or electronic pipettes. Many pipette types work by creating a partial vacuum above the liquid-holding chamber and selectively releasing this vacuum to draw up and dispense liquid. Measurement accuracy varies greatly depending on the style.

  (https://en.wikipedia.org/wiki/Pipette)

- Eppendorf Tubes :

  These tubes provide protection against unintentional opening during incubation and storage.

(https://online-shop.eppendorf.us/US-en/Laboratory-Consumables-44512.html)

- Vortex Mixer:

  A **vortex mixer**, or vortexer, is a simple device used commonly in laboratories to mix small vials of liquid. It consists of an electric motor with the drive shaft oriented vertically and attached to a cupped rubber piece mounted slightly off-center. As the motor runs the rubber piece oscillates rapidly in a circular motion. When a test tube or other appropriate container is pressed into the rubber cup (or touched to its edge) the motion is transmitted to the liquid inside and a vortex is created. Most vortex mixers have variable speed settings and can be set to run continuously, or to run only when downward pressure is applied to the rubber piece.

(*https://en.wikipedia.org/wiki/Vortex_mixer)*

- Lab Shaker:

A **shaker** is a device used in chemistry and biology laboratories to stir or mix substances, mainly liquids. Shakers are mainly used for mixing substances or incubating cell cultures in higher volumes.

(https://en.wikipedia.org/wiki/Shaker_%28laboratory%29)

- Magnetic Separator :

The multiwell plate is placed on the separator during the washes,so that the beads will surely hold on it due to its magnetical behavior.

- Luminex :

  Luminex's proprietary multiplex bead-based immunoassay testing platform simultaneously measures multiple analytes by exciting a sample with a laser, and subsequently analyzing the wavelength of emitted light.

  (https://en.wikipedia.org/wiki/Luminex_Corporation)

4. Experimental Procedure

For the particular experiment,we followed the following steps:

1. Resuspend the selected antibody-coupled microsphere sets (vortex and sonication for 10 seconds).

2. Mix coupled microspheres (stock concentration 5000 microspheres/ul) to a final concentration of 50 microspheres of each set/ul using as diluent Assay buffer (PBS,BSA 1%).

3. Mix biotinylated detection antibodies using as diluent Assay buffer(20ul of the detection antibody mix are needed for the sandwich ELISA reaction at step 14).The volume of each detection antibody needed for the reaction is different for each detection Ab and has been optimized.

4. Transfer 50ul of the microsphere mixture into each well of a flat bottom 96-well plate.

5. Place the plate on the magnetic separator ,wait for 1 minute and discard supernatant.

6. Add 100ul/well assay buffer.

7. Place the plate on the magnetic separator ,wait for 1 minute and discard supernatant.

8. Transfer 50ul of the sample or standard into the appropriate wells.

9. Cover plate with a plate sealer and shake it at maximum speed (800rpm) for 90 minutes at room temperature.

10. Place the plate on the magnetic separator,wait for 1 minute and discard supernatant.

11. Add 100ul/well assay buffer.

12. Place the plate on the magnetic separator,wait for 1 minute and discard supernatant.

13. Repeat steps 10-11.

14. Transfer 20ul of the detection antibody mix into each well of the plate.

15. Cover plate with a plat sealer and shake it at maximum speed (800rpm) for 60 minutes at room temperature.

16. Place the plate on the magnetic separator,add 100ul/well assay buffer and discard supernatant.

17. Add 100ul/well assay buffer.

18. Place the plate on the magnetic separator,wait for 1 minute and discard supernatant.

19. Prepare the PE mix,diluting 1:200 the stock (1mg/ml) SAPE into assay buffer.Make enough in order to add 50ul per well.

20. Add the freshly prepared ,photophobic (!) PE mix (50ul/well).

21. Cover plate with a plate sealer and shake it at maximum speed (800rpm) for 15minutes at room temperature.

22. Place the plate on the magnetic separator ,wait for 1 minute and discard supernatant.

23. Add 100ul/well assay buffer.

24. Place the plate on the magnetic separator ,wait for 1 minute and discard supernatant.

25. Remove the plate from the magnetic separator and resuspend the microspheres in 130ul/well assay buffer.

26. Cover plate with a plate sealer and shake it at maximum speed (800rpm) for 15minutes at room temperature.

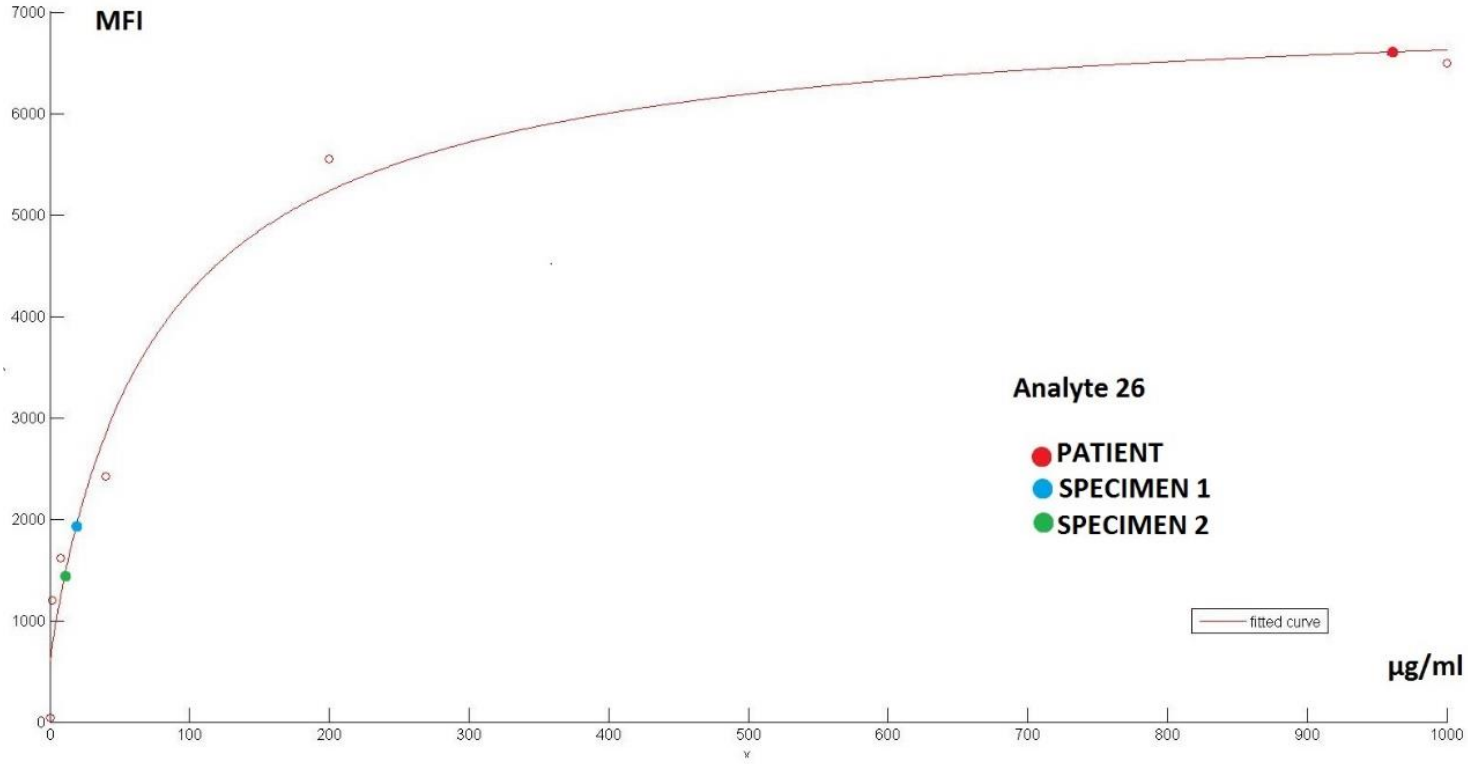
27. Remove the plate sealer ,place the plate in the Luminex instrument for measurement.

28. Analyze 100ul in the Luminex instrument according to the system manual.

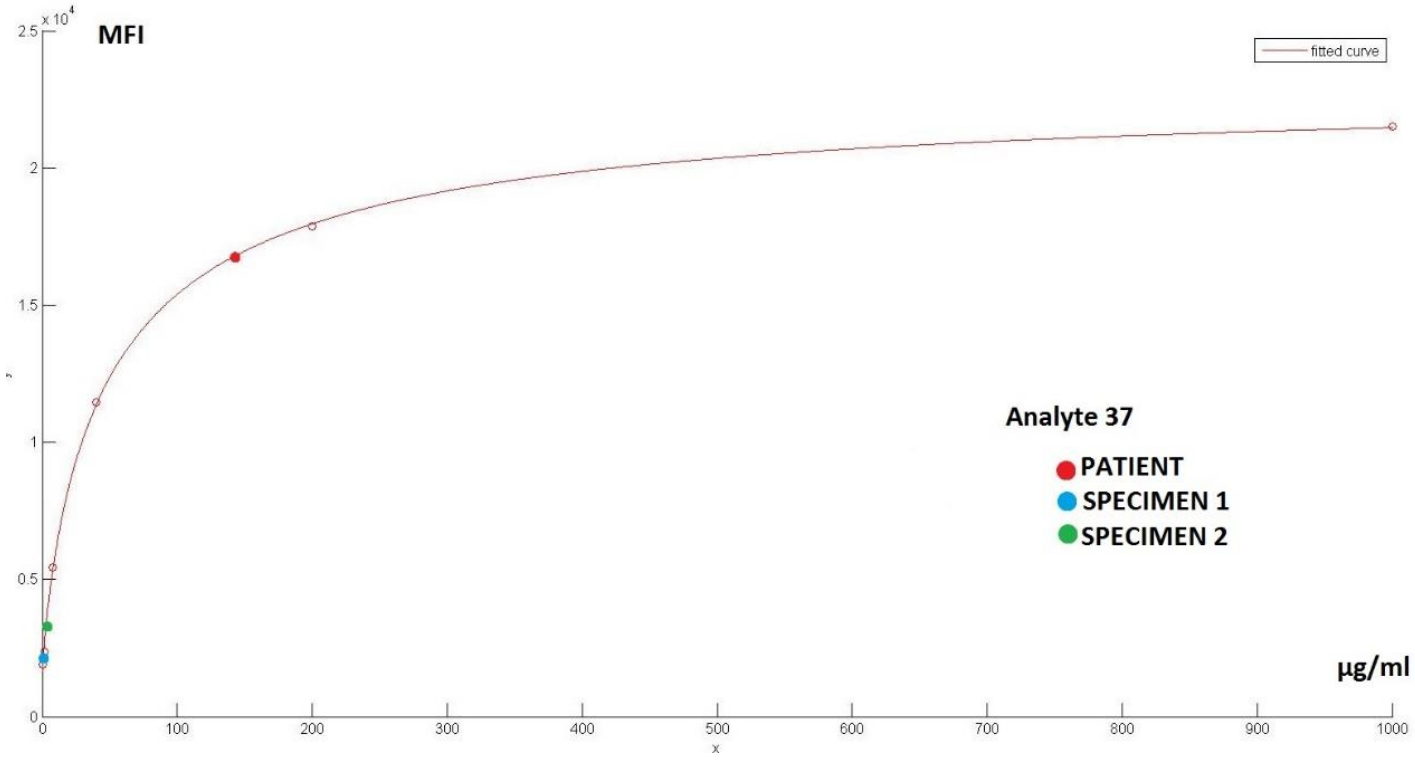
5. Processing and evaluation of the measurements

The data we got from the experiment are in MFI (MEDIAN FLUORESCENT INTENSITY). The bigger the value of the fluorescent intensity the bigger the concentration of albumin in the specimen. But we need to know the concentration of albumin in the specimens. In order to accomplish that we put dissolved albumin in some wells, dividing the concentration by 5 in each well.
With the results of the measurement of MFI we can now plot a curve showing the MFI/Concentration ratio. We managed that by using the L4P matlab file, that gives the appropriate sigmoidal shape to the curve. After plotting the curve we took the median of the three values of MFI we have for each specimen and find the respective ones of the concentration of albumin.

We used two different analytes for the measurements,so we completed the procedure for both analytes. The Analyte 26 lower led to lower values for MFI than the Analyte 37, which justifies the difference in the results.



Analyte 26 Curve



Analyte 37 curve

<u>Specimen 1</u>

Analyte 26

Concentration : 19,5 µg/ml

Analyte 37

Concentration : 1 µg/ml

<u>Specimen 2</u>

Analyte 26

Concentration : 11 µg/ml

Analyte 37

Concentration : 3 µg/ml

Patient

Analyte 26

Concentration : 143 μg/ml

Analyte 37

Concentration: 961 μg/ml

In conclusion,through the evaluation of the data we have acquired,there is only one patient since the upper limit of albumin in urine for a healthy person is 30 μg/ml.

6. Matlab Code

```
X = [ 1000 200 40 8 8/5 8/25]

Y = [  6492 5554.5 2418 1616 1195 38.5]

[cf G]=L4P (X, Y)

 hold on; plot(X,Y,'ro'); plot(cf,'r'); plot (19.5,1953,'+');plot (11,1452,'*');plot (961,6607,'^'); hold off


 X = [ 1000 200 40 8 8/5 8/25]

Y = [21517 17864 11452 5424 2375 1885 ]

[cf G]=L4P (X, Y)

 hold on; plot(X,Y,'ro'); plot(cf,'r');plot (1,2175,'+');plot (3,3299,'*');plot (143,16793,'^'); hold off
```

(First we plotted the curve without the points, then we found the exact values of concentration from the curve and at last we plotted the curves in the commands above.)

Sources

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